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# Uveal Melanomas in Labrador Retrievers

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Abstract

Canine uveal melanoma (UM) usually manifests as a slowly developing, darker pigmented and well distinguishable mass in the iris. Less than a third of them are considered malignant, which is much less than with other melanocytic cancers. In contrast, in humans, 90% of UM occurs in the choroid and half of the patients eventually develop aggressive and often lethal metastases. Understanding the disease process and genetic background in dogs might also help us further the knowledge and improve the treatment options of humans.

There is a hereditary component to the oncogenesis of the UM: the disease is more common in a Caucasian race and is also found in certain families. It is also more prevalent in certain dog breeds; Labrador Retrievers seem to be overrepresented. Several susceptibility genes have been identified in humans. One with the strongest association with UM is a tumor suppressor gene *BAP1*, which is dysfunctional or missing in nearly half of the human uveal melanomas. This gene is a so-called secondary driver of the UM and mutations in it spark the metastasizing process. There is a germline mutation of *BAP1* in fourth of Finnish UM families and these mutations are also connected to various other cancers. Moreover, *BAP1* shows over 98% protein product homology and almost 80% mRNA homology between dogs and humans, making it an appealing study target also for canines. Should a single variant account for high UM risk, a DNA test could be developed to be used in breeding and veterinary diagnostics.

In this work, I mapped the *BAP1* germline mutations of seven Labrador Retrievers with diagnosed uveal melanomas or melanocytomas. It was found that four dogs shared the same set of five heterozygous single nucleotide variants (SNV). One of the SNVs within exon 17 was synonymous, g.37,363,076G>A, p.(Ser721Ser), while the other four SNVs were intronic, residing close to exons 4, 10, 11 and 14.

In the future, variant comparisons with healthy Labradors are needed to study the role of the identified variants for the development of UM, as the SNVs now found could also just be a part of a common variation in the Labrador Retriever gene pool. To grasp a bigger picture of the UM tumor development, the tumors themselves should also be analyzed for somatic mutations. Moreover, when we know that the disease is likely affected by over a hundred genes, studying just one gene is unnecessarily self-restricting. Modern full genome sequencing techniques should be used for catching all the predisposing genes simultaneously.

Keywords
Dog, Labrador Retriever, eye, melanoma, uveal melanoma, cancer, tumor, genetics, heritability, *BAP1*

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Tiivistelmä

Koirien silmän suonikalvoston, eli uvean, melanooma (UM) on useimmiten tarkkarajainen ja tumma kohouma iiriksessä. Se kehittyy yleensä hitaasti ja lähettää etäpesäkkeitä harvemmin; vain alle kolmannes on pahanlaatuisia. Ihmispotilaista puolestaan tauti lopulta leviää noin puolella, usein aggressiivisena ja kohtalokkain seurauksin. Kasvava ymmärryksemme UM:n taustoista ja kehittymisestä koirissa voi auttaa ymmärtämään paremmin tämän monitahoisen sairauden syntyä ja kulkua myös ihmisissä. Spontaanisti UM:n sairastuvat lemmikkikoirat tarjoavat hyvän ja eettisen testikentän myös uusille hoitokeinoille.

Ihmisillä UM esiintyy selkeästi enemmän tietyissä suvuissa. On havaittavissa eroja myös koirarotujen kesken: labradorinnoutaja näyttäisi olevan yliedustettuna. Tämä viittaa perinnölliseen altistumiseen. Ihmisiltä on löydetty useita uveaalimelanoomalle altistavia geenejä. Suomalaisista perheistä, joissa esiintyy periytyvää UM:ää, neljänneksellä on ituradan mutaatio *BAP1* -geenissä. Itse kasvaimista tämä geeni puuttuu tai on toimimaton noin puolessa. *BAP1* -mutaatiot on myös yhdistetty moniin muihin syöpiin. Ihmisten ja koirien *BAP1* -geenit ovat hyvin samanlaisia; niiden tuottama mRNA on 80 %:sti samankaltaista ja niiden koodaamat proteiiniketjut ovat 98 %:n homologialla lähes identtisiä.

Tässä työssä kartoitin seitsemän iiriksen melanoomaa tai melanosytoomaa sairastaneen labradorinnoutajan *BAP1* -geenin koodaavan alueen mahdolliset sekvenssimuutokset niiden itusolulinjassa. Neljällä koirista ilmeni sama viiden eriperintäisen (heterotsygoottisen) emäsmuutoksen kaava: yksi synonyyminen variaatio eksonissa 17, g.37,363,076G>A, p.(Ser721Ser), sekä neljä varianttia intronialueilla lähellä eksoneita 4, 10, 11 ja 14. Lopuilla kolmella koiralla ei ollut lainkaan BAP1 sekvenssimuutoksia.
Seuraavaksi tarvitaan vertailevaa tutkimusta terveiden labradorinnoutajien *BAP1* -geenin sekvenssistä, jotta voidaan arvioida liittyvätkö havaitut variantit todellakin UM:n, vai onko kyseessä ainoastaan labradorinnoutajille ominaisista yhden nukleotidin polymorfismeista. Kasvaimen kehityksen kokonaiskuvan saamisesta olisi tutkimusta myös syytä jatkaa itse UM-kasvainten kudosnäytteiden kanssa: mahdolliset somaattiset variantit ovat yleisiä löydöksiä ja avaisivat UM:n alkuperää ja kehitystä laajemmin.

Yli sadalla geenillä arvioidaan olevan yhteys tähän tautiin, joten tutkimuksen rajoittaminen vain yhden geenin tarkasteluun on kapea-alaista. Resurssien salliessa olisikin hedelmällisempää lähestyä ongelmaa tulevaisuudessa koko genomin sekvensointien kautta sekä ituradan, että kasvainsolujen osalta.

Avainsanat
Koira, labradorinnoutaja, silmä, melanooma, syöpä, uveaalimelanooma, perinnöllisyys, genetiikka, BAP1

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# Contents

# 1 Review of the literature

Melanoma is a cancer of melanocytes, cells that produce melanin. Melanin is a dark pigment, and its amount dictates the hue of the skin and the eyes. Uvea is the pigmented layer of the eye that consists of iris, ciliary body, and choroid. Uveal melanomas (UM) arising from iris or ciliary body are called anterior, whereas those originating in choroid are called posterior. In dogs UM most commonly arises in the iris (Giuliano et al., 2002), whereas in humans the most usual location is posterior (Mobuchon et al., 2017). Despite differences between species, there are mutual benefits to be reaped from the increasing knowledge on disease process, genetics and treatment of both the human and canine UM.

In the first part of this work the current literature on the genetic basis of the UM is reviewed in both humans and dogs. In the experimental part genetic analysis on *BAP1*, a known tumor suppressor gene, is conducted on UM affected Labrador Retrievers (LR). *BAP1* is known to be missing or dysfunctional in many human uveal melanomas and therefore, represents also a relevant candidate gene for canine UM.

## 1.1 Uveal melanoma in humans

About 4 - 5% of all human melanomas are UM (Mobuchon et al., 2017). UM is the most common primary intraocular tumor in adult humans, its incidence being on average 5.1 new cases yearly per million people (Singh et al., 2011). The incidence seems to be constant with the pass of time as has been the mortality (Ezra et al., 2016). UM most often occurs at an older age, median age of UM diagnosis being around 62 years (Singh et al., 2011). At that age the yearly incidence is already more than twice the average (Kaliki and Shields, 2016).

UM can present either as a melanocytic or amelanotic mass, and can be local or diffuse (Kaliki and Shields, 2016). Shields et al. (2009) studied 8033 UM eyes and found that most of them, 7256, were posterior. Only 10 % were anterior, 492 occurring in the ciliary body and 285 in the iris. In their study, 45 % of the iridal melanomas occurred in the inferior quadrant of the iris and only 8 % in the superior quadrant. Interestingly, 11 % of the iridal melanomas were of a diffuse growth type, which had an almost four times higher relative metastasizing risk when compared with a non-diffuse UM. Only 3% of ciliary body and choroidal UM were diffuse.

In contrast to the melanoma of the skin, there does not seem to be a connection with the ultraviolet light (UV) exposure (Smit et al., 2020). However, an Australian study found a connection between ciliary body or choroidal melanomas and UV exposure (Vajdic et al., 2002). No connection could be drawn with iris melanomas and UV there neither (ibid.). Nevertheless, lighter eye and skin colors have been identified as risk factors, (Ferguson et al., 2016), and are thought to explain the four-fold higher incidence in the Northern European cultures when compared to the Southern European ones (Virgili et al., 2007). Also in Asia and Africa, the incidence is less than tenth of the average (Kaliki and Shields, 2016).

Metastases can be detected only in 4% of the UM patients at the time of the diagnosis (Smit et al., 2020). Eventually however, almost half of the patients develop metastases that are usually aggressive and lethal (ibid.). These occur most commonly in the liver and are sometimes only observed decades after removal of the primary tumor supporting theory of early-spreading long-dormant micro-metastases (Smit et al., 2020; Amaro et al., 2017). Bigger UM have higher propensity to metastasize (Shields et al., 2009). Other factors with good correlation to metastatic process are brown tumor color, location in the ciliary body, and high patient age (ibid.).

Four out of five American patients are still living after five years of diagnosis (Singh et al., 2011). In Europe this survival rate is ten percentage points lower, the difference most likely being explainable by the differing study inclusion criteria (Virgili et al., 2008). Bergman et al. (2003) studied 2997 UM patients over time span of 39 years and found

that 40% of them died within five years of diagnosis. More than half of the patients that perished during their study died because of UM (1023 out of 2003). Nevertheless, it is rather comforting that the anterior uveal melanomas are generally symptomless and even with secondary glaucoma only rarely painful in humans (Shields and Shields, 2009).

Perhaps surprisingly, also environmental factors, such as being exposed to cooking fumes, almost double the odds of contracting UM (Ge et al., 2012). Lynge et al. (2020) executed an ambitious case-control interview study to assess the connection of many rare cancers with occupational exposures. In addition to confirming the higher UM prevalence of cooks and welders, they also found an increased risk for dry-cleaners and launderers. They also made an interesting remark on the spectrum of the cooking gasses that might not be just a co-incidence: gas burners produce relatively low wavelength blue light, as do welding machines. However, the possible common exposing factor might as well be something related to the cooking/welding fume temperatures or chemicals.

## 1.2 Uveal melanoma in dogs

Contrary to humans, canine UM are most often anterior (Giuliano et al., 2002). Typical anterior UM manifests as a darker pigmented and well distinguishable mass in the iris, example of which is shown in the Figure 1.1. UM can also have a diffuse representation as in Figure 1.2, where the left iris of the dog has been thoroughly taken over by tumor: the iris is swollen, unevenly edged and rigid to light, and a secondary glaucoma has developed. In one study of 72 UM cases, third of the dogs developed glaucoma and uveitis, and about 10% manifested with hyphema (Wilcock and Peiffer, 1986).

Histologically canine UM usually consists of a mixture of spindle cells and epithelioid melanocytes (Zoroquiain et al., 2016). An example of a histological view of a malignant UM is shown in the Figure 1.3. Zoroquiain et al. (2016) suggested that the melanocytic lesions of the canine eye could be split into three categories: melanomas, melanocytoma-like melanomas and benign melanocytomas. When compared to melanocytomas, UM typically have bigger size, lighter color and higher mitotic activity, although in 18 of 28 UM they studied the mitotic index was less than the commonly used limit of 4 figures per

10 high-power fields (ibid.). For distinguishing melanocytoma-like tumors from UM using these three criteria worked flawlessly (ibid.). 29% of canine ocular melanomas can be considered to be malignant, which is considerably less than for other types of melanocytic tumors, their average malignancy being 70% (Gillard et al., 2013).

Canine UM seem not to metastasize as readily as human UM, possibly resulting from some difference between humans and dogs that prevents or slows further mutations. Studies have shown that neither the size, extension, nor mitotic index play a role in lifetime expectancy of the patients and recurrence after enucleation is rare (Giuliano et al., 2002). However, in a small follow-up study of 20 uveal melanoma dogs by Starkey et al. (2017), as many as eight of the dogs ended up having metastasis, seven of which could be found in the lungs but also skin, liver and systemic spread were observed. In that study the relationship between the primary tumor and metastases was not in all cases histologically verified. They could thus as well be of some other unrelated origin. This was the case with one of our study dogs that will be discussed later in the section 3.2. Other sites for metastasis have been reported as well, even prostate (Delgado et al., 2016), vertebra (Rovesti et al., 2001) and brains (Galán et al., 2009).

It is also possible that lack of metastases is in fact an observational bias due to the shorter life spans of dogs: in humans UM can metastasize even tens of years after removal of the primary tumor, so perhaps in dogs latent tumor cells just do not have enough time to gather required mutations to prosper elsewhere in the body.

Canine UM can be harmless for the individual during its life span and so knowing the genetic propensity for forming metastasizes might save some dogs from an unnecessary pre-emptive eye removal. In the future, knowing the genetic basis of these tumors might also open the door for targeted molecular therapies. Should a single variant account for high UM risk, could a DNA test be developed to be used in breeding and veterinary diagnostics.

**Figure 1.1:** Typical presentation of uveal melanoma in the left eye of the study dog MD4. A clearly discernible, dark, and outward bulging mass can be seen in the lateral part of the iris. Picture taken on the November 15th, 2018.

## 1.3 Uveal melanoma in Labrador Retrievers

According to a questionnaire study of UK dogs by Adams et al. (2010) almost a third, or 179 out of 574, LR died of some type of cancer. This was slightly higher than the average of all breeds, which in that same study was reported to be 27% of 15,881 deaths. The median age of death for LR was reported to be 12.25 years. Genetic predisposition of canine UM is suspected in some breeds, including the LRs, where Donaldson et al. (2006) found a single sire on both maternal and paternal sides of four anterior UM cases within five generations. Same sire was also found behind two cross breed UM cases.The underlying risk loci, however, have not yet been identified.

Possible genetic predisposition of UM in LR is also supported by studies that have reported LR as one of the breeds that is most commonly affected by UM. For example, in a treatment study of Cook and Wilkie (1999) 12 out of 23 dogs that had a melanocytic iridal mass were LR. Interestingly, eight of the affected LR were yellow bitches and three were black bitches. There was only a single yellow male amongst the affected dogs.

**Figure 1.2:** Untypical representation of UM in the left eye of the study dog MD3. Swollen iris has caused glaucoma and light rigidity of the iris has led to anisochoria. The diagnosis of melanoma could only be done in pathological examination. Picture taken on the April 4th, 2020.

Overrepresentation of females was also evident in the study of Giuliano et al. (2002), where 28 of 48 malignant ocular melanoma dogs, that is to say 58%, were bitches as were 112 of 181 ocular melanocytoma affected dogs (62%). These indicate a possible sex predisposition.

A microRNA study of Starkey et al. (2017) had 20 dogs with UM. Six of them were LR, four females and two males. About the same time Zoroquiain et al. (2016) performed a UM classification study, in which only 7 out of 64 dogs were LR. However, almost half of the dogs in their study, 24 out of 64, were reported to be of mixed-breed and could thus have LR ancestors. On the contrary, in an older study from 1985, none of the 12 uveal melanoma dogs were LR (Diters et al., 1983). A well-conducted epidemiological study with large cohorts would be needed to assess the true breed, sex and coat colour predisposition, together with other possible factors, for the development of UM in different dog breeds.

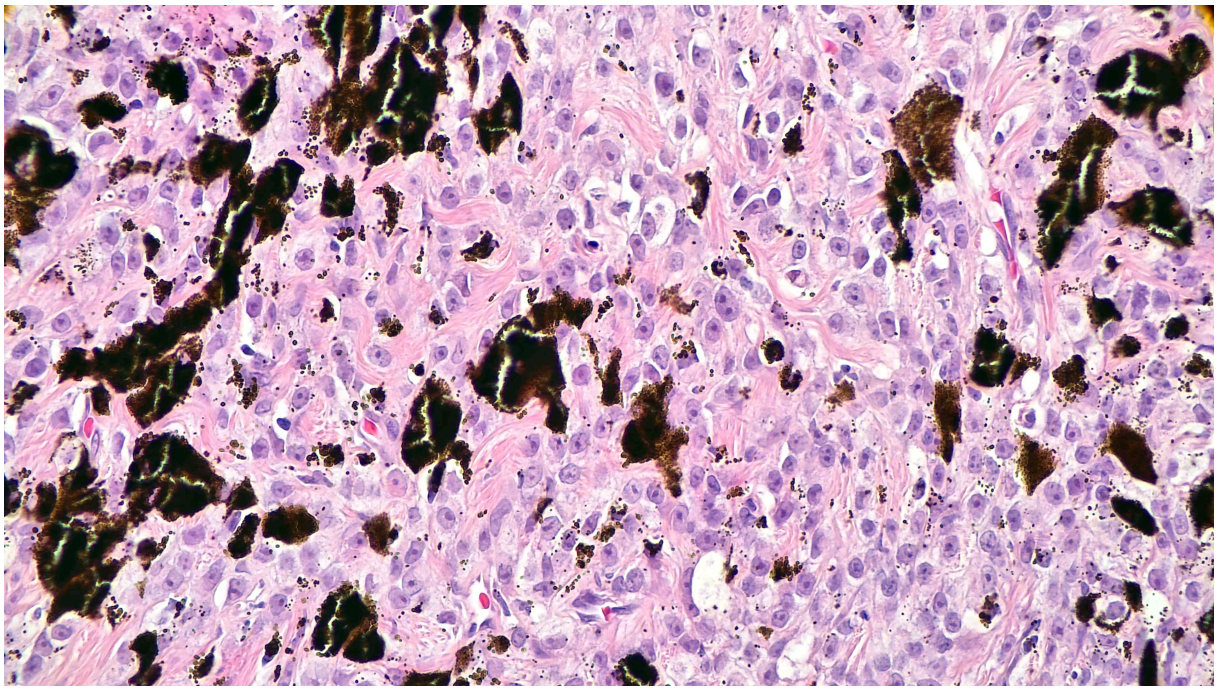

**Figure 1.3:** Histological view on a malignant UM showing unstructured pattern of oval cells of various sizes, and patches of dark pigment. This histological sample of study dog MD3 was prepared and stained by the University of Helsinki Pathology department.

## 1.4 Genetics of uveal melanoma

Genes have various roles in the cancer development. They can either be positive or negative regulators for the cell growth cycle and thus be named either proto-oncogenes, or tumor suppressors, respectively. Loss of function mutations in tumor suppressor genes, as well as gain-of-function mutations in a proto-oncogene may lead to cancer. Often both types of mutations as well as epigenetic dysregulation are needed (Senga and Grose, 2021; Hanahan and Weinberg, 2016). The mutations can either be inherited, or they might occur in the cancer cells themselves. Hereditary mutations that are manifested in all cells of the body are called germline mutations. Somatic mutations, on the other hand, denote non-hereditary changes in the DNA and initially arise in a single cell in the body. Somatic mutations can spread widely in the body by naturally occurring mitoses. This depends on the affected cell line and whether the mutation happens early or late in the life cycle of the organism. Mutations can also cause the cell to start dividing uncontrollably, which is when a cancer forms. These somatic mutations occur often due to outside influence such as exposure to radiation or carcinogenic chemicals.

**Table 1.1:** A list of genes most strongly associated with UM in humans following the MalaCards scoring: the higher the score, the higher the assumed association of the gene with the disease. Genes thought to be primary drivers of the UM oncogenesis have been bolded and those classified as secondary drivers have been underlined. Genes that were not found to have canine orthologs in the NCBI database (www.ncbi.nlm.nih.gov/gene) were omitted from the table, these included *RHPN1-AS1*, *PAUPAR*, *HOXA11-AS*, *MALAT1*, *UVM1* and *UVM2*. Added in the end of the table are some genes that are thought to have a connection to UM specifically in dogs.

| Gene | description | Function | Malacards score |
|---|---|---|---|
| *BAP1* | BRCA1-associated protein | removes ubiquitin from proteins | 758 |
| ***PLCB4*** | Phospholipase C Beta 4 | oncogene, linked to G$\alpha$11/Q pathway | 407 |
| ***GNAQ*** | guanine nucleotide-binding protein alpha Q | oncogene | 391 |
| ***GNA11*** | guanine nucleotide-binding protein alpha 11 | oncogene | 391 |
| ***CYSTLR2*** | Cysteinyl leukotriene receptor 2 | oncogene, linked to G$\alpha$11/Q pathway | 357 |
| *NUMB* | NUMB endolytic adaptor protein | tumor supressor | 153 |
| *MIR143* et co. | microRNAs | gene expression regulators | 57 |
| *SF3B1* | Splicing Factor 3b Subunit 1 | oncogene, metastasis predictor | 32 |
| *BRAF* | B-Raf proto-oncogene, serine/threonine kinase | CM oncogenesis, only anterior UM | 14 |
| *EIF1AX* | X-linked eukaryotic translation initiation factor 1A, | mutations lower the metastasis risk | 8 |
| *MBD4* | Methyl-CpG Binding Domain Protein 4 | DNA repair, tumor suppressor, might be a driver | - |
| *CDH1* | E-cadherin | upregulated in canine metastasizing UM | - |
| *FXR1* | FMR1 Autosomal Homolog 1, RNA binding protein | upregulated in canine metastasizing UM | - |
| *HTR2B* | 5-Hydroxytryptamine (Serotonin) Receptor 2B | mutated in 50% of human melanomas, upregulated in canine metastasizing UM | - |
| *LTA4H* | Leukotriene A4 Hydrolase | upregulated in canine metastasizing UM | - |

Curiously the genetic background of UM differ notably from other melanomas (Hendricks et al., 2018; Weyden et al., 2020; Stei et al., 2016). This might have to do with the immune-privileged status of the eye, that leads to a unique micro-environment and immune responses (Zhou and Caspi, 2010).

A selection of genes that are most strongly associated with uveal melanomas are shown in Table 1.1. They are organized in the order of disease-gene association scoring by Malacards (www.malacards.org, Rappaport et al. (2016)), which sorts them by their estimated level of association to the UM in humans. Altogether MalaCards database suggests 116 UM associated genes, only a few of which are discussed here.

Current understanding for UM development in humans is that it is first initiated by a somatic mutation in one of the primary driver genes: *GNA11*, *GNAQ*, *CYSTLR2* or *PLCB4*. After this, mutations in a secondary driver gene, *BAP1*, *SF3B1* or *EIF1AX* are accelerating the tumour development (Shain et al., 2019). Secondary drivers are usually mutually exclusive and UM with a mutated *BAP1* have a poorer prognosis, than do those with mutations in either *SF3B1* or *EIF1AX* (Xiangyu et al., 2017).

A lot of effort has been put into identifying possible genetic factors on predicting disease severity and metastatic risk. Onken et al. (2012) have proposed a gene expression profiling assay that in their study succeeded in malignancy classification of 446 of 459 cases. They profiled gene-expressions from fine needle aspiration biopsies of the lesions and found that in malignant tumors *CDH1*, *ECM1*, *HTR2B*, and *RAB31* were upregulated, whereas *EIF1B*, *FXR1*, *ID2*, *LMCD1*, *LTA4H*, *MTUS1*, *ROBO1*, and *SATB1* were downregulated.

Compared to other cancer types, UM commonly have low mutational burden of less than one single nucleotide variation (SNV) per Mb (Smit et al., 2020). However, in contrast to many other cancer types, they have been shown to keep evolving genetically and their metastases harbor more mutations than the primary cancer does (Shain et al., 2019).

### 1.4.1 *BAP1*

*BAP1* is a gene coding the BRCA1 associated protein 1, which is a deubiquitylating enzyme. The gene is located in chromosome 3 in humans and 20 in dogs. Its protein product binds to the breast cancer type 1 susceptibility protein (BRCA1) and acts as a tumor suppressor. All its modes of action are still not completely known, but it has roles in at least cell cycle and growth regulation, DNA damage repair, epigenetic regulation, inflammatory response and cell metabolism (Bergmann et al., 2018).

Nearly half of the UMs in humans are missing, or have a mutated *BAP1* gene (Harbour et al., 2010; Figueiredo et al., 2020). Some acquire this mutation hereditary, but it can also occur somatically as a local driving mutation behind the oncogenesis and spreading of the UM. In a Finnish study fourth of the families (4 out of 16) associated with UM also had a pathogenic germline mutation of *BAP1* (Repo et al., 2019). In the same study only 1.9% of all UM patients (8 out of 432) had a germline *BAP1* mutation. The loss of *BAP1* promotes metastasis formation and its function is lost in majority of the metastatic UM, but the exact mechanism is still unclear (Smit et al., 2020; Harbour et al., 2010). It is curious however, that persons with the *BAP1* mutation in the germline have a better prognosis, than do those with mere somatic mutations, suggesting an alternative tumor suppression process active on these individuals (Smit et al., 2020).

It is understood, that *BAP1* and its germline mutations play a part in causing the human tumor predisposition syndrome, TPDS (Bergmann et al., 2018; Young et al., 2020). In addition to lurking behind UMs, BAP1-TPDS is also associated with the heightened risk of cutaneous melanomas, mesotheliomas, renal and basal cell carcinomas and likely many other types of cancers as well (Bergmann et al., 2018).

Recently, Uner et al. (2021) came into conclusion that *BAP1* mutations occur very early in the UM development. In their study, the tumors that had *BAP1* mutations had the biggest overall masses as well as the largest individual tumor cells. Their large cohort study also showed that UM metastasized over six times sooner (2.4 vs. 16 years) when they possessed a debilitating *BAP1* mutation.

When compared across species, *BAP1* shows over 98% protein product homology and almost 80% mRNA homology between dogs and humans (Jama et al., 2018), making it an appealing study target. On the other hand, Jama et al. (2018) already studied the connection of canine melanoma and *BAP1* immunohistochemistric loss. They found no connection between loss of *BAP1* and UM, as of the eight UM samples studied all were positive in *BAP1* labelling. Same applied to 81 of the 82 other studied melanomas, including skin, digit, periocular and oral melanomas. Although *BAP1* was not incapacitated, it does not rule out some other form of gene damaging. In their view, however, it would be more probable that the mechanisms on melanoma development in dogs were independent from *BAP1*.

### 1.4.2 Other genes

In addition to *BAP1*, also several other genes have been associated with UM in humans. For example, deregulation of GN$\alpha$11/Q pathway is strongly linked to UM oncogenesis with over 95% of human UM victims showing somatic mutations in either *GNAQ* or *GNA11* (Smit et al., 2020). These mutations, or alternatively somatic mutations in *CYSTLR2* or *PLCB4*, seem to be so-called primary drivers and mainly affect the initiation of the cancer (ibid.).

Mutations in *BAP1*, *SF3B1*, and *EIF1AX* weigh in later in the cancer development. SF3B1 is a subunit in a spliceosome that is needed in splicing of pre-mRNA and *EIF1AX* codes for a translation initiation factor. Their mutations are usually mutually exclusive and quite prognostic in nature: somatic *BAP1* mutations could be used as a marker for poor prognosis, whereas mutations in *SF3B1* and *EIF1AX* could indicate better prognosis (Decatur et al., 2016).

Methyl-CpG Binding Domain Protein 4 gene, *MBD4*, is one of the newest genes associated with UM. Its protein product is a tumor suppressor that takes part in DNA repair. Mutations in it are quite rare both in germline and somatically, but when occurring can cause the cancer to mutate with increased frequency (Derrien et al., 2020). These genomic changes can then be used to deduct the timeline for the individual UM

development (Rodrigues et al., 2019).

New potential driver genes are continually identified: In a genome-wide association study (GWAS) of 259 human UM patients it was found that higher *CLPTM1L* germline expression correlates with the oncogenesis (Mobuchon et al., 2017). Later, another GWAS of 590 UM patients also identified a germline risk locus in the DNA repair gene *TDP1* (tyrosyl-DNA phosphodiesterase 1) (Thomsen et al., 2020). Whole exome sequencing (WES) study by Abdel-Rahman et al. (2019) identified significantly increased amount of pathogenic germline mutations in *SMARCE1*, *PALB2* and *MLH1* in connection with human UM. Additionally, their study hinted that variants in other cancer predisposition genes, namely *MSH6*, *CHEK2*, *ATM*, *BRCA1* and *CTNNA1*, might also play a role.

Malho et al. (2013) investigated in a small study (19 dogs of which 6 were LRs) whether 14 genes known to differ between metastasizing and non-metastasizing UM in humans would also be prognostic indicators in canines. As a result, four of the genes used for assessing metastatic potential in humans also had statistically significant changes in their expressions in dogs, namely *HTR2B*, *FXR1*, *LTA4H* and *CDH1*.

Shain et al. (2019) also mention uveal melanomas on humans to often have changes in chromosomal numbers: extra copies of 6p and 8q, and losses of 1p, 3, 6q, 8p and 16q have been encountered. Monosomy of chromosome 3 is observed in about half of human UM and also has a strong correlation with metastasizing (Tschentscher et al., 2001; Scholes et al., 2003). As in humans *BAP1* resides in the chromosome 3, monosomy of this chromosone also influences oncogenesis and behavior of UM by alternating *BAP1* expression.

### 1.4.3 MicroRNAs

MicroRNAs are tiny stretches of single-stranded RNA, only around 20 nucleotides long. They are non-coding but take part in gene expression regulation by influencing mRNA stability and translation. A recent review of Li et al. (2020) listed 13 microRNAs that are associated with UM in humans, many of which are oncogenic and upregulated in affected individuals, whilst others are tumor suppressors and downregulated, respectively.

Starkey et al. (2017) identified 9 microRNAs having altered expression when comparing dogs with metastasizing UM with those whose cancers were non-metastasizing. As microRNAs are very stable and can be extracted from bodily fluids such as tears, blood, milk or vitreous humour, they might prove to be a good biomarker and prognostic indicator for this disease already during its early stages (Li et al., 2020).

## 1.5 Dogs as models for human disease

Dogs have been selectively bred for tens of thousands of years to form phenotypically diverse breeds that are genetically quite homogenous, yet sharply distinct from each other (Ostrander et al., 2000). Inbreeding enhances expression of recessively inherited trades and diseases, which facilitates their genetic mapping. Studying genetics of dog breeds can thus be said to be analogous to studying isolated human groups and their specific diseases, such as those in Finnish people (ibid.). Besides demonstrating similar looks and behavior, individuals of the same breed also often share susceptibility for various breed-specific diseases. Of about 450 known hereditary diseases in dogs around half have corresponding human diseases (Tsai et al., 2007). Finding the underlying genetic susceptibilities on canines can help in understanding similar disease processes in humans (Lindblad-Toh et al., 2005; Hytönen and Lohi, 2016).

In pet dogs, the tumor development processes are quite similar to humans in that tumors in them arise naturally in contrast with the studies done on laboratory mice (Prouteau and André, 2019). Pet dogs share the environment with their humans and are thus cheaper to access than laboratory mice. Moreover, pet dogs also make more ethical model animals as no reproduction, tests or killing is taking place solely for the research purposes. Tsai et al. (2007) also pointed out that humans have a strong will to cure diseases of pet dogs, making them great model candidates when studying and testing new treatments for diseases as new cutting-edge treatments will then also be rapidly available for dogs.

Breed dogs make especially good models for genetical diseases as they often have well documented family lineages. We also share a lot of physiology and genetics with

dogs, especially when compared with the laboratory mice. As an example, cutaneous melanomas are not rare on canines and provide ample of opportunities to study genetics, treatments and etiologies (Weyden et al., 2020; Gillard et al., 2013). Studying UM in the dogs might well shed new light also into understanding the evolution and progression of human UM (Zoroquiain et al., 2016).

## 1.6 Therapies

In canine patients the most common method to treat UM is enucleation, which often offers sufficient clinical benefit as it prevents painful secondary manifestations such as obstructive glaucoma. In humans, UM is often both more aggressive and more lethal and removal of the eye is undesirable, hence a lot of effort is put into finding other efficient means to fight the disease. Dogs are usually considered to fare well in life with only one eye or even when totally blind. However, enucleation in dogs might not be absent of adverse consequences: bumping into objects on the blind side as well as erroneously estimating jumps (e.g., to a car or over ditches) are described by owners. Thus, finding treatment options that do not include losing the eyesight would be welcomed also in the veterinary side.

Traditional cancer treatment methods consist of chemotherapy and radiotherapy in addition to surgery. Local delivery of radiation can be performed with plaques of radioactive material inserted in or close-by the cancer tissue. This so-called plaque brachytherapy is the mainstay of UM therapy method on humans (Stålhammar, 2020; Echegaray et al., 2017). Radiation therapies however are not without their problems and their application in a way that is safe also for the owner of a pet dog might be challenging. Radiation therapies predispose dogs also to keratitis, retinal damage, uveitis, choroidal atrophy, optic nerve degradation and other side-effects (Ramos et al., 2019). More elegant and specific methods are in development.

One example of a more straightforward eye-saving method is diod laser photocoagulation, which has been used for over 20 years to treat canine suspected iris melanomas with good results (Cook and Wilkie, 1999; Spiess, 2012). On humans proton beam radiotherapy

has provided good results with minimal damage to adjacent healthy tissues (Desjardins et al., 2012). In a French study of 1406 proton beam treated UM patients the local recurrence rate was only 4% during a 5 year follow-up (Dendale et al., 2006).

Already 20 years ago, an adenovirus was used in a preclinical study to deliver human angiostatin (AdK3) directly into an anterior uveal tumor of a Great Dane and two Beagles (Andrawiss et al., 2001). Various further oncolytic virus treatments are currently being developed. One example showing promise is the E. coli cytosine deaminase being vectored into cancer cells by oncolytic Herpes Simplex Virus type 1 (Liu et al., 2020). Another interesting technique is photoimmunotherapy that utilises Human Papillomavirus (HPV) capsids with affinity to the cancer cells. These papillomavirus-like particles can be used in delivering cytotoxic substances directly into the cancer cells. When combined with photosensitizing agents that become cytotoxic only under influence of light, the tumor can be targeted very specifically (Kines et al., 2018). A clinical study is currently being conducted on usability of this method to treat primary choroidal melanoma in humans (NCT03052127, clinicaltrials.gov/ct2/show/NCT03052127).

Programmed cell death receptor 1 (PD-1) and its ligand (PD-L1) antibodies are promising targets for cancer therapies (Ribas and Wolchok, 2018; Zoroquiain et al., 2018). A normally functioning PD-1 receptor interacting with PD-L1 ligands regulates the T cell immunoresponse. Monoclonal antibodies suppressing this interaction thus end up strengthening the immunoresponse targeted at the cancer cells and have been used with success for treatment of various human cancer types, with antibodies against PD-1 showing the most promise (Duan et al., 2020). They have also been used for canines to treat malignant mucosal melanoma (Maekawa et al., 2017).

Antibodies for another T-cell surface protein, cytotoxic T lymphocyte-associate protein 4 (CTLA-4) are being successfully used for treating cutaneous melanomas (Ribas and Wolchok, 2018). Combining them with PD-1 and PD-L1 blockers ameliorates the overall results further (Rotte, 2019). Although cutaneous melanoma has been one of the first success stories for these antibody therapies, it seems that UMs use other means to hide from the immune system as they express much less of both PD-1 and PD-L1 than do

cutaneous melanomas, and are thus not as susceptible to these antibody therapies (Javed et al., 2017).

COX-2 receptors of the cancer cells could also be possible therapeutic targets. In human UMs, higher COX-2 expression is associated with metastasis and poorer prognosis (Cryan et al., 2008). COX-2 inhibitors have thus been considered as an adjuvant therapy for many cancers but have lost some popularity due to adverse cardiovascular effects. Celecoxib however shows great promise as an addition to human cutaneous melanoma treatment regime, but some of its beneficial pro-apoptotic effects seem to be unrelated to its COX-2 inhibitory properties (Filip, 2020).

A recent review by (Szweda et al., 2020) summarizes the current knowledge of the role of COX-2 on various cancers of dogs and cats. The exact mechanisms behind anti-tumor effect of non-steroidal anti-inflammatory drugs remain unknown, but also on canines seem to be independent of the COX-2 inhibition (Yoshitake et al., 2017). A study of 66 canine UMs revealed affinity to monoclonal COX-2 antibodies in 86% of the tumors and, moreover, the expression was dependent on the malignancy of the tumor, being increasingly expressed with more aggressive melanomas (Esposito et al., 2016). Paglia et al. (2009) studied COX-2 expression in 71 canine globes, four of which were healthy while others harboured malignant UM (n = 34), uveal melanocytomas (15), mixed melanocytic neoplasms (8, these had malignant UM and melanocytoma), or suffered from some other nonneoplastic disease (10). More than twice as often did the eyes with malignant UM show COX-2 expression in the iris (2 of the eyes) or in the ciliary body (20), when compared with the other groups (11 out of 37, none of which had any iridal COX-2 expression). However, the difference was not so evident that the COX-2 expression could be used to differentiate between benign and malignant lesions (ibid.).

Encouragingly, a commonly used canine pain medication firocoxib has been shown to enhance the remission rate of urinary bladder transitional cell carcinoma especially in combination with chemotherapy drug cisplatin (Knapp et al., 2012). As approved coxib products for dogs are readily available, well tolerated, and have a proven effect on pain control, there is little reason not to take them into consideration when drafting cancer

therapies.

MicroRNA's might also provide an interesting new therapy target. They play a part in tumor oncogenesis as well as in metastasizing. Epigenetic drugs such as genistein or 5-aza-2'-deoxycytidine appear to have some promise in treating the uveal melanomas by regulating the expression of some involved microRNAs (Li et al., 2020).

The field of cancer therapies is advancing fast and wide, and also other forms of immunotherapies are emerging: lymphokine-activated killer cell therapy, vaccines, gene therapy, oncolytic virotherapy and bacteria activated immunotherapy among others might prove effective in the future (Almela and Ansón, 2019; Yang et al., 2018).

# 2 Hypothesis and aim of the study

LRs are overrepresented amongst the dogs affected with UM and are thus suspected to be genetically predisposed to the disease. Germline mutations in *BAP1* are strongly associated with UM in humans. *BAP1* could also be a risk gene for canine UM. This hypothesis is tested here by screening *BAP1* coding and splicing regions for mutations in seven LR that have been diagnosed with UM or melanocytoma.

# 3 Materials and methods

## 3.1 Ethical statement

All experiments were approved by the Animal Ethical Committee of the County Administrative Board of Southern Finland (ESAVI/6054/04.10.03/2012, ESAVI/7482/04.10.07/2015, ESAVI/343/04.10.07/2016) and relevant guidelines and regulations were followed. Dog owners gave their written consent prior to participation.

## 3.2 Study cohort

Seven LRs that had been diagnosed with melanoma or melanocytoma of the eye, were included in the study, see Table 3.1. Phenotyping was performed from histological tissue samples by a pathologist and/or by an ophthalmologist during an eye examination. All affected dogs were born in Finland and represented diversely both the working and show lineages, into which the breed is genetically divided (Lampi et al., 2020). Hence, the affected dogs were not closely related apart from MD1 and MD2 that were littermates.

General health information of the study dogs was limitedly available, with the exception of MD3 and MD4 that were owned by the author. They are shown in Figures 3.1 and 3.2 before and after their eye enucleations. Closer views of their UM eyes were shown earlier in Figures 1.2 and 1.1. MD3, in addition to her UM, was also found to be affected by a choroidal melanoma in her other eye. Moreover, she had diffuse carcinomatous epithelial metastases in her lungs diagnosed in a post-mortem examination. At a young age, she also had a benign histiocytoma in the skin of her hind leg. During the last months of her life she started slowly accumulating malignant effusion in her pleural space. At

**Table 3.1:** A summary of the affected dogs studied. Shown is the gender (F for female and M for male), as well as the coat color and description of the ailment. OS, left eye; OD, right eye; HPF, high-power field; XX, either eye.

| Study ID | gender | colour | Melanoma type |
|---|---|---|---|
| MD1 | F | black | XX: malignant melanoma in iris |
| MD2 | F | black | OD: malignant melanoma in iris |
| MD3 | F | black | OS: malignant melanoma in iris and ciliary body, infiltrating into choroidea, and, in lesser extent, to sclera. Secondary glaucoma |
| MD4 | F | yellow | OS: benign iridal melanocytoma. |
| MD5 | M | brown | XX: melanocytoma or melanoma in iris. Mitosis count (<2/HPF) refer to melanocytoma, while diffuse growth type to melanoma. |
| MD6 | M | yellow | OS: malignant melanoma in iris. |
| MD7 | F | yellow | OS: malignant melanoma in iris. |

the age of 13 years, almost a year after her left eye was removed, she presented with a sudden violent seizures and was euthanized. No signs of any brain lesions could be found in obduction.

MD4 lived almost two years after her left eye was enucleated and was euthanized at the age of 15 years due to her dwindling eyesight and worsening osteoarthritis. A post-mortem examination was performed, revealing an invasive clear cell carcinoma in her liver. Both MD3 and MD4 also had Meibomean gland adenomas.

## 3.3 DNA extraction

From each study dog, a peripheral EDTA blood sample (1-3 ml) had been collected and deposited into the Canine Biobank of the University of Helsinki. Extraction of the genomic DNA from the white blood cells was done using an automated Chemagen robot (PerkinElmer Chemagen Technologie GmbH, Baesweiler, Germany). Purity and concentration of DNA in the samples were measured with Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), the Nanodrop ND-100 UV/Vis Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) or the DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, Delaware, USA).

**Figure 3.1:** Author's own dogs, study subjects MD3 (black bitch on the left) and MD4 (yellow bitch on the right) representing both the guide-dog/show dog lineages (MD4, registered as Opas Eevi) as well as the hunting lineage (MD3, registered as Jummi-Jammin Faye Dunaway). One can note the enophthalmus on the left eye of the MD4. This has led to the showing of the nictitating membrane, or third eyelid, and is often a sign of pain. The enucleation was performed three days after this picture was taken. Vaasa, Finland, January 19th, 2019.

## 3.4 PCR and Sanger sequencing

Nominal protein and mRNA sequences for the *BAP1* gene were extracted from the National Center for Biotechnology Information, NCBI, database (www.ncbi.nlm.nih.gov). Genomic sequence was retrieved from the USCS database (genome.ucsc.edu). CanFam 3.1 annotation was used in all genetic analyses. Primer3 software, primer3.ut.ee (Koressaar and Remm, 2007), was used for designing the primers to cover the coding and splicing regions of the *BAP1* gene. Primer sequences are listed in the Appendix. Primers were provided by the Sigma Aldrich (St. Louis, USA). PCR amplification was made using the Biotools DNA Polymerase (Biotools B&M Labs, S.A., Valle de Tobalina, Madrid,

**Figure 3.2:** Study subjects MD3 (black bitch on the left) and MD4 (yellow bitch on the right) after their UM affected eyes had been enucleated. The hair around MD3s' enucleated eye has not yet grown back to its full length. Myrskylä, Finland, June 1st, 2020.

Spain) according to manufacturer's instructions. PCR products were run on 1% agarose gels to confirm expected product size. They were purified using ExoSAP process with Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA, USA). Samples were capillary sequenced at the FIMM Institute for Molecular Medicine. Finally, the Unipro UGENE (v38.1, Mar 26 2021) software was used to analyse variants within the *BAP1* coding and splicing regions.

## 3.5 *In silico* prediction on variant pathogenicity

Pathogenicity of the identified missense variants were predicted with in silico tools PolyPhen-2, (Adzhubey et al., 2010), and PROVEAN, (Choi and Chan, 2015). These programs predict variants pathogenicity by scores calculated examining the altered amino acid conservation among different species.

# 4 Results

Sequencing the whole coding and splicing regions of the *BAP1* gene in genomic DNA samples from seven UM affected LRs identified altogether five variants, which are displayed in Table 4.1. One of the variants was located within the coding region and four were within introns. The coding variant was synonymous.

Of the studied dogs, four, namely MD1, MD4, MD6 and MD7 shared the same variant pattern: the synonymous c.2163G>A; p.(Ser721Ser) -variant and four intronic variants: c.123-35T>C, c.931+54T>G, c.1,116+32C, and c.1,890+118G. Interestingly, dogs MD1 and MD2, two females from the same litter, did not share any of the variants.

**Table 4.1:** Mutations found in the *BAP1* gene of the affected dogs. The dogs are represented by codes listed in Table 3.1. First column shows the position counting base pairs from the forward 5' end of the chromosome 20. Last column shows the exon closest to the mutation. All the variations found were heterozygous. MD2, MD3, and MD5 were of wild type with respect to these variations.

| Position & change | Affected dogs | closest exon |
|---|---|---|
| g.37,356,892; c.123-35T>C | MD1, MD4, MD6, MD7 | Exon 4 |
| g.37,359,694; c.931+54T>G | MD1, MD4, MD6, MD7 | Exon 10 |
| g.37,360,332; c.1,116+32C>T | MD1, MD4, MD6, MD7 | Exon 11 |
| g.37,362,318; c.1,890+118G>A | MD1, MD4, MD6, MD7 | Exon 14 |
| g.37,363,076G>A; c.2163G>A; p.(Ser721Ser) | MD1, MD4, MD6, MD7 | Exon 17 |

# 5 Discussion

In this work, a common pattern of single nucleotide variations in germline *BAP1* gene was found in four out of seven LR with UM. This might be revealing a predisposing heritable variation behind canine UM, which would be quite exciting especially as some previous studies did not find a loss of expression of *BAP1* in UM (Jama et al., 2018). However, a more probable explanation for the observed mutational pattern is that it could be just a typical SNP pattern for the breed and not specifically connected to the UM. To find the truth, we would need to compare these results with the gene sequencing results of healthy LR *BAP1* gene and then verify the findings on a larger population.

*BAP1* is a tumor suppression gene and its mutations in humans spark development of many different cancers (see Section 1.4.1). *BAP1* has been connected with a tumor predisposition syndrome, TPDS, which is most often characterized by uveal melanoma (Bergmann et al., 2018). As at least two of the study dogs, MD3 and MD4, had multiple different cancers during their lifetime, it would be tempting to suggest something similar to be going on in canines. However, a study with only seven affected dogs and only one studied gene is far too small and weak for drawing any such conclusions.

This study was performed on a single gene and on seven individual dogs. In view of this, the observed pattern seems surprisingly clear and further studies are warranted. Whole genome sequencing studies would allow looking simultaneously at all the other over hundred suspected genes behind UM, and perhaps also to find some totally new ones. Larger cohort of both UM dogs as well as matching numbers of healthy control dogs would improve the weight of the results significantly. Since the completion of the laboratory works described here, another two Finnish UM LR were identified, so there is

already material available for a slightly wider study.

The somatic mutations of the tumor tissues themselves should also be studied for better understanding the oncogenesis, development and spread of UM. Moreover, widening the study to other breeds would bring more power and breadth to it. Lastly, it would have been interesting to compare the pedigree of the study dogs with each other, but alas, currently there exists no pedigree dog database in Finland that would be available for research purposes.

# 6 Conclusions

In this work, we have studied the *BAP1* gene on Labrador Retrievers and whether individuals suffering from uveal melanoma might, as do humans, have pathogenic variations of that gene. *BAP1* is a commonly mutated gene in human UM patients and as it is highly homologous to the respective canine gene, it provided a natural target for our study.

A common variation pattern was indeed found in the majority (4 out of 7) of the study dogs, but it remains to be verified whether this actually relates to the UM. The observed pattern might as well be breed specific and further studies are needed to clarify this.

UM is a potentially deadly disease in humans. Dogs provide a naturally occurring readily available model for the research and treatment of the disease. Growing understanding of the underlying disease process will enable new diagnosis and treatment methods to benefit both species.

In this work, a preliminary study material and data has been gathered of Finnish LR suffering from UM. Moreover, multitude of veterinarians, owners and breeders have been contacted and informed about this research. Thus, a ground has been laid for identification and recruitment of further subjects. Preliminary *BAP1* screening performed here paves the road for further studies and can be used as a guide in formulating new research strategies on the subject.

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# Appendix A

# Primers

**Table A.1:** Primers used for the Sanger sequencing PCR. These were designed with the free web based Primer3 tool (Koressaar and Remm, 2007). Exon 17 was so long that it was sequenced in three parts.

| exon(s) | direction | sequence |
|---|---|---|
| 1 - 3 | Forward | 5'-gccggaccctggagaatc-3' |
| | Reverse | 5'-tgggattcctactctacctcc-3' |
| 4 | Forward | 5'-gaaagaggccagggtagagg-3' |
| | Reverse | 5'-tctctaatcaccactcctgct-3' |
| 5 | Forward | 5'-tgatgagttttgttgtccagct-3' |
| | Reverse | 5'-acctgacaaagtcctaacaaagt-3' |
| 6 - 8 | Forward | 5'-ccaggcctattttccagagc-3' |
| | Reverse | 5'-ggaggcccaagatctaagct-3' |
| 9 - 10 | Forward | 5'-ccctgccaatttgttcgctt-3' |
| | Reverse | 5'-ccgctctccatgtttcaagg-3' |
| 11 | Forward | 5'-gctatgtctggtgggtgtct-3' |
| | Reverse | 5'-ggtaacagagagacttggcca-3' |
| 12 | Forward | 5'-gtgggctctggagtaactgg-3' |
| | Reverse | 5'-actcagtaggtgctcagtgt-3' |
| 13 | Forward | 5'-accaagtatgaggagctgca-3' |
| | Reverse | 5'-aagaagacccttcagagtgc-3' |
| 14 - 15 | Forward | 5'-cacttggtggacggaggag-3' |
| | Reverse | 5'-catcatagttgtgggtccgc-3' |
| 16 | Forward | 5'- ggtcacctttctcccagct -3' |
| | Reverse | 5'- agcagccacccactcaat -3' |
| $17^1$ | Forward | 5'- ctcaggcctcttatggtgct -3' |
| | Reverse | 5'- tcaggaagctggctatgtca -3' |
| $17^2$ | Forward | 5'- cagctccgggaatgggac -3' |
| | Reverse | 5'-aatactgaggggctgggc -3' |
| $17^3$ | Forward | 5'- ctagtaggggtgggatgagc -3' |
| | Reverse | 5'- tgatcacaggaccttgggc -3' |

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